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(54) Technique for visualization of genetic material

(57) A technique for the visualization of mammalian genetic material in living germ cells, one-cell fertilized eggs and pre-implantation embryo cells is disclosed. The cells are incubated with a fluorescent dye and the stained DNA is visualized by exposing the cell to ultraviolet light. A process of genetic manipulation is also disclosed which comprises transferring DNA into the above-mentioned cells and embryos in which the transferred DNA and/or the cell's own DNA is visualised by the fluorescence technique.

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SPECIFICATION

Technique for visualization of genetic material

This invention relates generally to staining techniques for biological genetic material. More specifically, the method of this invention provides a unique means of visualizing (1) the pronuclei of living pronuclear cells, including ova and one-cell fertilized eggs (2) the condensed DNA in the heads of spermatozoa, and (3) the nuclei of pre-implantation embryos. Visualization of the genetic material in these types of living cells is critical for further research and development of *in vitro* fertilization and maturation, gene and nuclear transfer capabilities and subsequent cloning of domestic animal embroys. Heretofore, pronuclei and sperm DNA have not been seen in living, viable mammalian cells, except for rodents and humans where the cytoplasm of these cells is clear. In more mature embryos, the area of the nuclei may be somewhat discernable, but the cells' smaller size makes it impossible to visualize individual nuclei.

Advanced genetic improvement and selection techniques continue to be sought in the field of animal husbandry. With specific reference to dairy cattle, for example, significant increases in milk production have been seen with the wide scale use of genetically superior sires and artificial insemination. Dairy cows today produce nearly twice as much milk as they did 30 years ago. Further genetic improvement can be accomplished by the multiplication of superior or genetically manipulated embroys by cloning. Embryos may be cloned by (1) replacement of pronuclei or nuclei in a fertilized egg cell with donor pronuclei or with nuclei from an early multicellular embryo or (2) transfer of a valuable gene into the pronucleus or nucleus.

Gene transfer requires the pronucleus or nucleus of the recipient cell to be visible so that DNA fragments containing the desired gene sequences can be transferred by micromanipulation directly into one of the pronuclei or the nucleus. Nuclear transfer, on the other hand, requires the nucleus of the male and female pronuclei to be visible so that they can be removed from the fertilized cell in exchange for a nucleus from the desired clonal line. Techniques such as these involving the manipulation of genetic material were developed in research with murine embryos in which the cytoplasm of the pronuclear and embryo cell is clear, permitting visualization of the nuclear material in living cells by light microscopy.

The situation in bovine and porcine gametes and pre-implantation embryo cells is quite different. The cytoplasm of these cells is dense and granular and contains thick lipid droplets. The genetic material is obscured and cannot be visualized by light microscopy. Conventional nuclear stains are not vital stains; that is, they require that the cells be fixed and cleared prior to staining. Bovine and porcine gametes and pre-implantation embryo cells therefore normally have been examined in fixed, cleared and nonliving specimens.

As discussed above, genetic improvement techniques such as gene and nuclear transfer require the selective staining of the nuclear or pronuclear material in living pronuclear or embryo cells. These techniques, together with a host of laboratory research tasks, are made possible by the technique of this invention which provides a visualization method with the necessary selectivity and which can be used with living cells.

SUMMARY OF THE INVENTION

The technique described herein enables the male and female pronuclei to be visualized in living mammalian cells, even in those species in which the density of the cell cytoplasm normally obscures the pronuclei. Visualization of pronuclei in living cells has not previously been reported, except rodent and human cells. The technique also provides a means for visualization of the nuclear material of multi-cell embryos, making it possible to count the number of cells present in a developing embryo after each cleavage as well as manipulate the genetic material for gene and nuclear transfer.

Visualization is accomplished by exposure of the living cells to a stain or dye which is specific for double stranded DNA but which does not preclude the continued viability of the cell. Fluorescent stains, such as DAPI or the bis-benzimidazole compounds, are used. The stain is added to the culture medium containing gametes, one-cell fertilized eggs or multi-cell embryos. After incubation, which can be for as short a time as several minutes, the genetic material can be visualized by exposure to ultraviolet light. The pronuclear or nuclear structures appear as stained areas within the cytoplasm. The intensity varies from very faint to bright fluorescing.

An overall object of this invention is to enable and facilitate laboratory research tasks such as gene and nuclear transfer techniques, and studies of the patterns and progress of *in vitro* maturation of oocytes, and *in vitro* fertilization and embryo culture.

One of the principal objects of this invention is to provide a technique for the visualization of the pronuclei of living mammalian pronuclear cells.

It is a more specific object to permit visualization of the male and female DA or pronuclei of living mammalian gametes and one-cell fertilized eggs, bovine and procine in particular.

It is a further object to provide a like technique which will allow for the visualization of nuclear material of multi-cell embryos.

A related object is to provide a means for the differential staining of the genetic material and the cytoplasmic contents.

Still another object is to provide a means of staining the genetic material in living germ and embryo cells in a manner which is likely to allow maintenance of the health and viability of the cells.

In addition, it is desired to provide a stain which will not cause excessive damage or alteration to the genetic material.

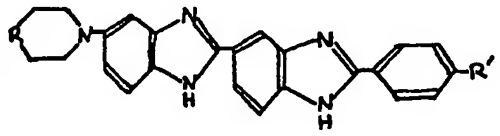
DETAILED DESCRIPTION OF THE INVENTION

The method of this invention provides a technique for the differential staining of certain genetic material which never before has been visible in living cells. The fluorescent stains used in this method selectively stain the genetic material of the cells, without requiring the cells to be fixed and cleared. The selectivity of this staining technique provides a powerful research tool which will facilitate further investigation of fertilization, embryological development and gene and nuclear transfer in domestic animal embryos.

The stains of this method can be used to selectively stain the genetic material of any species. However, the technique finds its highest value with those species in which the cytoplasmic materials (e.g., lipids) obscure the pronuclear or nuclear material in the living state. Examples of considerable interest and importance to the animal breeding industry are cattle and swine. Application of the technique to other research or domestic animals is contemplated as well.

It has been found that certain fluorescent stains used according to this method will achieve the dual objectives of selectivity of staining and continued viability of the cells. Fluorescent stains which are so-called "vital stains," that is, which do not require the cells to be fixed and cleared and which do not interfere with the continued development of the cell, may be useful in this procedure. In addition, the stain should be of the type which is incorporated into the cell and which is taken up by the double stranded DNA in living germ or embryo cells.

Stains belonging to the class of bis-benzimidazole fluorescent dyes have been found to be particularly useful in this method. This class of compounds has the basic structure:



A series of stains in this class is available from Hoechst AG, Frankfurt, Germany. Compounds in this series include:

Hoechst No.	R	R'
32020	$CH_3-N<$	-Cl
32021	$CH_3-N<$	$-OCH_3$
33258	$CH_3-N<$	-OH
33342	$CH_3-N<$	$-OC_2H_5$
33662	$C_2H_5-N<$	$-OCH_3$
34580	$CH_3-N<$	$-N<^{CH_3}_{CH_3}$
38312	$H-C<$	-Cl
38317	$H-C<$	$-NH_2$

Studies by S. A. Latt et al., "Spectral Studies on 33258 Hoechst and Related Bisbenzimidazole Dyes Useful for Fluorescent Detection of Deoxyribonucleic Acid Synthesis," Journal of Histochemistry and Cytochemistry, Vol. 23, pp. 24–33 (1976), demonstrate the similarity of the series

of compounds listed above and disclose that these compounds are useful in the detection of DNA synthesis in somatic cells.

The Examples that follow demonstrate that Hoechst stain Nos. 33258 and 33342 are useful in the technique disclosed herein. The chemical name of Hoechst stain No. 33258 is 2-[2-(4-hydroxyphenyl)-6-benzimidazoyl]-6-(1-methyl-4-piperazyl)-benzimadazole. The chemical name of Hoechst stain No. 33342 is 2-[2-(4-ethoxy phenyl)-6-benzimidazoyl]-6-(1-ethyl-4-piperazyl)-benzimadazole. It is expected that the related bis-benzimidazole compounds will all be useful for the selective visualization of genetic material in living germ or embryo cells. Of the two compounds actually tested, Hoechst stain No. 33342 significantly outperformed Hoechst No. 333258 and therefore is the preferred reagent. It has been noted, however, that the cells of the various species will take up different stains with differing efficiency. For example, Hoechst No. 33258 was taken up rapidly by mouse embryos, but quite slowly by bovine embryos.

Other fluorescent stains meeting the requirements described above, that is, DNA-binding fluorochromes which may be used as vital stains, are expected to be useful with this method as well. Examples include, but are not limited to DAPI (4'6-diamidino-2-phenylindole), and DIPI (a compound similar to DAPI).

The method by which the genetic material in gametes or embryo cells is stained essentially entails exposure of the cells to a suitable fluorescent stain, incubation and exposure to ultraviolet light. The stain dosage and the incubation period can be varied, depending on the stain used and the purpose for which visualization is desired.

No false positives have been found using this staining technique; that is, the procedure has not been found to significantly stain materials other than nuclear or pronuclear DNA. It has been noted, however, that overstained cells will exhibit some background staining. The technique has been found to result in some false negatives; that is, some cells failed to fluoresce which were found by other methods to contain nuclei or pronuclei. It is not considered that this will hinder or compromise the usefulness of the technique in either a laboratory or commercial setting.

Egg cells (oocytes), one-cell fertilized eggs or pre-implantation embryos are obtained from mature female animals by conventional means which will preserve the health of the cells. Sperm cells (spermatozoa) are obtained from the ejaculate of mature male animals or from excised testes, epididymis or part of the male duct system. Germ cells or gametes (oocytes and spermatozoa) are haploid cells which will unite to form a fertilized egg. The fertilized egg (containing the male and female pronuclei) then undergoes syngamy (the union of the male and female pronuclei to produce a zygote nucleus), followed by a series of cell divisions. As the embryo undergoes a series of mitotic divisions, it develops through the morula stage to the blastocyst stage, when tissue differentiation and specialization occur. Ultimately, this leads to formation of the fetus and placenta, which become implanted on the uterine wall.

It is the embryo in the pre-implantation stages, as well as the germ cells and one-cell fertilized eggs, which are the primary focus of this invention. These very early cells are the subjects of intensive research efforts. The gene and nuclear transfer techniques described above utilize fertilized egg cells and early embryo cells. The technique has proven useful in other embryological research as well. For example, it enables researchers to study the development of *in vitro* fertilized eggs, e.g., the symmetry of nuclear and cytoplasmic division, the appearance of a single nucleus in each blastomere, the numer of cells in the embyro after each division, etc. In addition, it is useful for studying the health and motility of spermatozoa.

The germ or embryo cells preferably are placed in culture medium appropriate for maintaining the health and viability of the cells. The preferred culture medium for a given application, e.g., for cells of a particular species and at a particular developmental stage, will be known to one skilled in the art. Several suitable media are given in the Examples. Sperm may be examined in semen, in semen with conventional extenders such as egg yolk or milk extender, or in culture medium.

The stain preferably is used in solution. This allows for more precise regulation of the dosage, as well as allowing very dilute strengths of the stain to be used. It is believed that lower dosages may have less tendency to cause genetic damage to the cell.

A stock solution of the stain may be prepared using any suitable solvent; that is, any solvent for the stain which will not adversely affect the living cells. Examples include, but are not limited to Dulbecco's Phosphate Buffered Saline (DPBS), TALP Whittens or Eagles media, or any other media or solvent compatable with the continued viability of the cells. The stain stock solution preferably should be stored at refrigerated temperatures, preferably about 0°C to 4°C, in order to reduce the possibility of contamination. However, it should be noted that freezing generally will not be desirable as it causes the salts to precipitate out of the solution.

The strength of the stain stock solution may be varied, as desired. A stronger solution will stain genetic material in a shorter period, whereas a weaker solution will require a longer incubation period before the material fluroesces. In addition, the stain uptake rate varies with the species, with bovine and porcine cells requiring a much longer incubation period than murine cells. As noted above, lower dosages of the stain may be preferred to maintain the

health and viability of the cells.

For example, using a stock solution of 20 μg stain per ml DPBS, as little as 1 μl in 49 μl of media (50 μl total volume) containing mouse one-cell embryos resulted in distinct fluorescence of pronuclei and at least one polar body within 15 minutes. Using bovine one-cell embryos and 20 μg/ml stain stock solution at a dosage of 2 μl in 48 μl of culture media containing embryos, polar bodies fluoresced within 30 minutes, while the pronculei were not visible for 1 to 2 hours. As can be seen with reference to the Examples, the dose and incubation period may be varied as desired for various experimental or other purposes.

The stained structures may be visualized microscopically by exposure to ultraviolet light. Light at an excitation wavelength of about 350 nm and emission about 460 nm has been found to be suitable. Nuclei and pronuclei appear as stained blue areas within the cell cytoplasm. Sperm have no discrete pronuclei; the DNA is condensed and packed into the head of the sperm where is may be visualized by this method. Where pronuclei are referrred to, it is meant to include the DNA of sperm, unless otherwise specified. Depending on the amount of stain taken up by the DNA, which is a function of dose and time, the intensity will vary from very faintly to brightly fluorescing.

The following Examples are given for illustrative purposes only and are not meany to limit the invention described herein except as defined by the appended claims. The stock solutions and culture media used in the Examples were prepared as indicated below.

Preparation of Dulbecco's Phosphate Buffered Saline (DPBS)

Dulbecco's Phosphate Buffered Saline (DPBS) was prepared as follows:

Solution #1	8.00	gm NaCl
	0.20	gm KCl
	1.15	gm Na_2HPO_4
	0.20	gm KH_2PO_4
	800.00	ml deionized distilled water
Solution #2	0.1	gm $CaCl_2$
	100.0	ml deionized distilled water
Solution #3	0.1	gm $MgCl_2$
	100.0	ml deionized distilled water

Each solution was steam autoclaved separately at 120–125°C for 20 minutes and then cooled. The three solutions were mixed in a one liter volumetric flask, and the total volume adjusted to one liter with autoclaved deionized distilled water. The DPBS stock solution was refrigerated at about 4°C.

When DPBS was used to wash cells, 5 mg (0.1ml) gentamycin sulfate stock solution first was added to 100 ml DPBS. The gentamycin stock solution was 50 mg gentamycinSO_4 per ml NaCl.

Preparation of Stain Stock Solutions

Stain stock solutions using Hoechst No. 33342 were prepared in the following concentrations:

10 μg/ml DPBS
20 μg/ml DPBS
60 μg/ml DPBS

Stain stock solutions using Hoeschet No. 33258 was prepared in the following concentrations:

10 μg/ml DPBS
20 μg/ml DPBS

The stain stock solutions were stored at 4°C.

Preparation of TALP (Tyrode-Albumin-Lactate-Pyruvate) Medium

Preparation of stock solutions for use in making up TALP medium was as follows:

Ingredient	Quantity	In:
NaCl	9.210 gm	1.0 l H_2O
KCl	1.237 gm	100 ml H_2O
$CaCl_2(2H_2O)$	1.332 gm	100 ml H_2O
$MgCl_2(6H_2O)$	2.436 gm	100 ml H_2O
$NaHCO_3$	1.403 gm + 1 mg phenol red	100 ml H_2O
Glucose	5.310 gm	100 ml H_2O
Na Lactate	1.0 ml DL-Lactic Acid*	
$NaH_2PO_4(H_2O)$	28.0 mg	**

*DL-Lactic acid, sodium salt as 60% syrup (Sigma Chemical Co.). The 1.0 ml lactic acid syrup was rinsed into the 35 ml H_2O until it was in solution. One drop phenol red was added. The pH was adjusted to approximately 7.4 with NaOH (overshoots may be corrected by adding a very small drop of lactate).

**The 28 mg $NaH_2PO_4(H_2O)$ was mixed with 10 ml of the Glucose stock solution as prepared above.

The ingredients for each stock solution were mixed, Millipore filtered (0.22 μ pore size) into sterile bottles, and refrigerated at about 4°C.

The TALP medium was made up as needed by mixing the following quantities of stock solutions:

Stock	Stock Conc.	Conc. in Medium		Quantity
NaCl	157.0 mM	114.00 mM		to 100 ml
KCl	166.0 mM	3.16 mM		1.90 ml
$CaCl_2(2H_2O)$	120.0 mM	2.00 mM		1.70 ml
$MgCl_2(6H_2O)$	120.0 mM	0.50 mM		0.41 ml
$NaHCO_3$	167.0 mM	25.00 mM		15.00 ml
$NaH_2PO_4(H_2O)$	20.5 mM	0.35 mM)	together	1.70 ml
Glucose	295.0 mM	5.00 mM)		
Na Lactate	150.0 mM	10.00 mM		6.70 ml

The indicated quantities were mixed together with sufficient NaCl to bring the final volume to 100 ml and 6.5 mg penicillin (100 i.u./ml) and 1.0 mg phenol red were added. The resulting mixture was Millipore filtered (0.22 μ pore size) into a sterile bottle.

On the day of use, the TALP medium was supplemented as follows, depending on the desired use:

1. *Fertilization Medium*–6 mg Bovine Serum Albumin (BSA) (fatty acid free) and 10 μl Na pyruvate stock per ml TALP was added and Millipore filtered. (BSA from Sigma Chemical Co.) or
2. *Maturation Medium*–to 4.5 ml TALP, 500 μl fetal bovine serum (10%), 50 μl Na pyruvate stock and 10 mgFSH/ml TALP were added and Millipore filtered.

Preparation of Whitten's Medium

Ingredient	Quantity
NaCl	514.0 mg
KCl	36.0 mg
KH_2PO_4	16.0 mg
$MgSO_2(7H_2O)$	29.0 mg
NaHCO	190.0 mg
Na Pyruvate	3.5 mg
Ca Lactate ($5H_2O$)	53.0 mg
Glucose	100.0 mg
K Penicillin	8.0 mg
StreptomycinSO_4	5.0 mg

The medium was made up by adding the listed ingredients to a 100 ml volumetric flask. Next, 0.37 ml of 60% Na Lactate syrup (Sigma Chemical Co.) and 0.1 ml of 1% phenol red were added. The final volume was adjusted to 100 ml with deionized distilled water. The medium then was Millipore filtered (0.22 μ pore size) and stored at 4°C. On the day of use, the medium was supplemented with Bovine Serum Albumin (BSA) (Sigma Chemical Co.) by adding 1.5 mg BSA per ml medium.

EXAMPLE I

Mouse embryos were used in this Example to verify the specificity of the stain for genetic material in living one-cell embryos because the pronuclei of this species are visible in the unstained state. The embryos were obtained from mature female mice which has bred the previous night. The animals were sacrificed by cervical dislocation and the ovaries and oviduct excised. The oviduct was dissected free and one-cell embryos recovered by locating them in the oviduct as a bulging area and puncturing the oviduct wall with fine needles and fine scissors. The cumulus cells were removed by adding 100–200 μl hyaluronidase solution (10 mg/ml DPBS) to the area of the dissected oviduct and allowing it to sit for 10 minutes. The one-cell embryos were recovered by pipetting, and were washed three times in a solution of 3 mg Bovine Serum Albumin (BSA)/ml DPBS before adding them to Whittens medium. The BSA was purchased from Sigma Chemical Co.

Individual drops of Whittens medium were placed on a plastic culture dish. The drops were scratched to cause them to adhere to the plate and then were overlaid with oil. The one-cell embryos then were placed in the drops. The pronuclei were visualized using light microscopy before being stained according to the method of this invention.

The 20 μg/ml strength stain stock solution of Hoechst No. 33342 was used for this Example. It was desired to give the one-cell embryos dosages of 0, 1 μl, 2 μl, 5 μl or 10 μl of stain stock solution in a total medium-embryo-stain volume of 50 μl per drop. It therefore was necesary to place the medium-embryo drops on the slides in sizes to accommodate the desired stain dosage. That is, 48 μl medium-embryo for 1 μl stain dosage, 48 μl medium-embryo for 2 μl stain dosage, etc. The embryos were stained at 37°C, pH 7.2 in 5% CO_2-in-air. They were left in the stain while being visualized.

The pronuclei and polar bodies of the one-cell mouse embryos were visualized using Nikon epi-fluorescence attachment No. TMD-EF for Inverted Microscope DIAPHOT-TMP (Nikon). This system uses a high pressure mercury lamp. The UV filters used were by Nikon. This system permits visualization with light and fluorescence simultaneously so that it was possible to positively identify the areas of fluorescence as the pronuclei and polar bodies.

All of the 66 embryos stained in this manner showed distinct fluorescence of both pronuclei and at least one polar body within 15 minutes of initial exposure to the stain, regardless of dosage. Frequently, only one polar body was evident as the second polar body degenerates with time.

Next, 24 one-cell mouse embryos were stained in the same manner as above, using the low dose only (1 μl stain solution with 49 μl medium-embryo drop). Twenty-three of the 24 fluoresced within 5 minutes after staining.

EXAMPLE II

The procedures of Example I were repeated using one-cell hamster embryos and a dosage of 10 μl stain solution per 50 μl total drop volume. The first polar body stained after 15 minutes exposure to the stain. The pronculei did no fluoresce until 30 minutes exposure. Up to 45 minutes exposure was required for complete visualization of the two pronuclei and at least one polar body in each egg. As in Example I, both polar bodies did not always stain.

EXAMPLE III

The procedures of Example I were repeated using matured bovine oocytes, one-cell and 2-cell embryos. Ovaries were obtained from a slaughterhouse. The oocytes were aspirated from the follicles. The cumulus cells were removed prior to staining with hyaluronidase and pipetting as in Example I.

Some of the recovered oocytes were used for staining, as described below, and some were used for *in vitro* fertilization. The oocytes were incubated for 24 hours at 39°C in TALP maturation medium and then incubated with ejaculated bull semen in TALP fertilization medium. One-cell fertilized egg cells were recovered after the 24 hour incubation, washed in DPBS-gentamycin solution and resuspended in fresh TALP fertilization medium. Two-cell embryos were prepared in the same manner, with recovery after 48 hours incubation with sperm and fertilization medium, and then washed and resuspended in fresh fertilization medium.

Stain stock solution with a concentration of 20 μg Hoechst No. 33342 per ml DPBS was used for this Example. The procedures of Example I were used with stain dosages of 2 μl and 5 μl stain per 50 μl total volume of medium-embryo-stain. The stain-culture mixtures were incubated at 39°C, mounted on glass slides under cover slips, and visualized under ambient conditions.

Polar bodies were visible within 30 minutes after the stain was added. The pronuclei of the oocytes and one-cell embryos and the nuclei of the two-cell embryos were visible after one to two hours or more.

The stained cells then were mounted, fixed and cleared to provide a cross-check on the chromatin present. The slides with the stained embryos and oocytes were placed in coplin jars containing approximately 40 ml of acetic acid:ethanol (1:3 (vol/vol)) and allowed to clear overnight at room temperature. Chromatin was stained with 1% aceto-orcein (wt/vol) (1% orcein stain in 60:40 water-acetic acid solution). The chromatin was visualized by light microscopy using Nemarsky and phase optics.

It was found that approximately 15% of oocytes and early one-cell embryos contained metaphase plates or pronuclei although they had failed to fluoresce upon exposure to the bis-benzimidazole stain. With the two-cell embryos, no instances were seen in which the nucleus failed to fluoresce. The stain did not give any false positives; all embryos which fluoresced were found to contain chromatin when evaluated by the second method. The chromatin was present in one of the following forms: (1) scattered or metaphase plate for oocytes (including germinal vesicles for the immature oocytes), (2) pronuclei for fertilized egg cells, (3) nuclei for embryo cells, or (4) condensed sperm heads which penetrated the oocyte but did not undergo normal decondensation to form the male pronucleus.

EXAMPLE IV

The basic procedures of Example III were repeated using porcine oocytes, one-cell fertilized eggs and two-cell and four-cell embryos. The oocytes were recovered from bred sows by surgical flushing of the oviducts and suspended in TALP maturation medium. The 60 g/ml strength stain stock solution of Hoeschst No. 33342 was used according to the procedures of Example I with stain dosages of 0, 1 μl, 5 μl and 10 μl per 50 μl total volume of medium-embryo-stain. These mixtures were incubated at 39°C for 3 hours.

The metaphase plates and polar bodies of oocytes which had not been fertilized and fertilized eggs stained very distinctly, with pronuclei appearing less distinct. In addition, sperm heads which had penetrated the zona of oocytes showed fluorescence. The nuclei of two-cell and four-cell embryos showed very distinct fluorescence. It was found that the 10 μl dosage resulted in high levels of background staining.

The stained cells were mounted, fixed and cleared as in Example III, except that they were cleared in acetic acid:ethanol for 48 hours. The results were similar to those in Example III. About 40% of the oocytes and one-cell embryos had failed to fluoresce, although no false positives were observed. The multi-cell embryos were routinely stained with the Hoechst stain.

EXAMPLE V

The *in vitro* development of embryos exposed to Hoechst fluroescent stain No. 33342 was evaluated with mouse embryos at the two-cell and morula stages. The embryos were obtained and prepared by the method described in Example I. They were stained with 10 μl doses of stain solution (20 μg/ml concentration, resulting in a dosage of 0.2 μg per 50 μl total drop volume.

The embryos were exposed to the stain for 15 minutes. Next, the embryos were washed three times in DPBS-gentamycin and cultured in Whitten's medium.

Of 26 two-cell mouse embryos exposed to the stain, 16 or 65% developed to the morula stage. This compares with 21 of 26 controls (80%) exposed to no stain, i.e., incubated for 15 minutes with 10 μl doses of DPBS alone.

When morula-stage embryos were exposed to the stain, 23 of 44 (51%) developed to

blastocysts. Of the controls (DPBS only), 23 of 31 (74%) developed to blastocysts.

Example VI

The basic procedures of Example I were repeated, using Hoechst No. 33258 (10 μg/ml and 20 μg/ml stain stock solutions) to stain one-cell mouse embryos. The dosages were 0, 5 μl, and 10 μl in 50 μl total medium-embryo-stain volume. The embryos were stained under ambient conditions for one-half hour before being examined. The pronuclei and polar bodies fluoresced and were positively identified using light microscopy as in Example I. It was found that the 10 μl dosage of this stain did not routinely cause overstaining or background staining.

Example VII

Bovine spermatozoa were stained with Hoechst stain No. 33342 in order to assess sperm motility and tracking patterns. Freshly ejaculated bull semen was washed with DPBS and centrifuged. The resulting pellet was suspended in TALP medium and exposed to 20 μg/ml concentration Hoechst No. 33342 stain stock solution at a dosage of 3 μl/50 μl. The medium-sperm-stain mixture was incubated for 15 minutes at 39°C and observed.

Following staining, the fluorescing sperm were visualized using the Nikon fluorescence system described in Example I. A camera in the microscope's optical system was used to take color time lapse photographs at exposures of from about 10 to 20 seconds. The tracks produced by the fluorescent DNA on the developed photographs were analyzed as to rate and pattern of sperm motility. The procedure was repeated with the addition of cumulus-oocyte complexes to the medium, and the resulting photograph was analyzed as to the effect of these cells on sperm motility rate and pattern.

Example VIII

This example was conducted to observe cleavage in porcine embryos of various stages. Embryos were obtained from the reproductive tracts of superovulated sows 40–120 hours after the onset of estrus. The reproductive tracts were recovered after slaughter and were flushed in the laboratory to remove the embryos. Embryos recovered ranged from one-cell (48 hours) to multicell morula (120 hour) embryos. Following two washings in a DPBS-BSA solution, the embryos were either cultured in Whitten's medium (control) or stained and then cultured in Whitten's medium.

The 60 μg/ml strength stain stock solution of Hoechst No. 33342 was used for this Example. The embryos were incubated for one hour at 39°C in either 1, 5 or 10 μl stain solution per 50 μl. This was followed by three 30-minute washings in clean DPBS (1.5 mg/BSA/ml). The washed embryos were cultured for 24 hours in Whitten's medium (1.5 mg/BSA/ml) and then were evaluated for development (completing cleavage division). The results are shown in Table 1.

Table 1

Stage	Dose			
	Control	1 μl	5 μl	10 μl
1 or 2 Cell	6/12(50%)	--	2/14(14%)	1/12(8.3%)
4 Cell	28/36(77%)	17/27(62%)	14/37(37%)	--
Multicell	15/16(94%)	--	13/23(56%)	--

The principles, preferred embodiments and modes of operation of the present invention have been described in the foregoing specification. The invention which is intended to be protected herein however, is not to be costrued as limited to the particular forms disclosed, since these are to be regarded as illustrative rather than restrictive. Variations and changes may be made by those skilled in the art without departing from the spirit of the invention.

CLAIMS

1. A method of staining the DNA of living mammalian cells selected from the group consisting of germ cells, one-cell fertilized eggs and pre-implantation embryo cells, comprising:
(a) contacting said cells with a fluorescent stain, and
(b) incubating until said DNA fluoresces when exposed to ultraviolet light.
2. The method of Claim 1 in which said cells or eggs are in culture medium.
3. The method of Claim 1 or 2 in which the fluroescent stain is selected from the group comprising DAPI, DIPI and bis-benzimidazole compounds.
4. The method of Claim 3 in which said stain is used in solution.

5. The method of Claim 3 or 4 in which the dosage of said stain is from about 0.001 μg to about 10 μg per 50 μl culture containing said mammalian cells.

6. The method of Claim 1 substantially as hereinbefore described.

7. A mammalian cell selected from the group consisting of germ cells, one-cell fertilized eggs and pre-implantation embryo cells, the cell having its DNA stained in accordance with the method of any of Claims 1 to 6.

8. A living cell or clone derived from the cell of Claim 7.

9. A method of staining pronuclei in living mammalian pronuclear cells comprising:
(a) contacting living mammalian pronuclear cells with a flurorescent dye, and
(b) incubating untilthe pronuclei fluoresce.

10. The method of Claim 9 in which said living mammalian pronuclear cells are bovine or porcine one-cell fertilized eggs.

11. The method of Claim 9 or 10 in which said cells are in culture medium.

12. The method of Claim 9, 10 or 11 in which the fluorescent dye is selected from the group comprising DAPI, DIPI and bis-benzimidazole compounds.

13. The method of Claim 12 in which the said dye is in solution.

14. The method of Claim 12 or 13 in which the dye dosage of said stain is from about 0.001 μg to about 10 μg per 50 μl culture drop containing said pronuclear cells.

15. The method of Claim 9 substantially as hereinbefore described.

16. A living mammalian pronuclear cell having its pronculeus stained in accordance with the method of any of Claims 9 to 15.

17. A living cell or clone derived from the cell of Claim 16.

18. A process for removing genetic material from a living mammalian germ cell, one-cell fertilized egg or pre-implantation embryo cell comprising:
(a) suspending living cells or eggs in a medium,
(b) contacting the suspension with a fluroescent dye,
(c) incubating until the genetic material fluoresces upon illumination with ultraviolet light, and
(d) removing said genetic material from said cells using micromanipulation techniques.

19. The process of Claim 18 in which said pronuclear cells are bovine or porcine one-cell fertilized eggs.

20. The process of Claim 18 or 19 in which the fluroescent dye is selected from the group comprising DAPI, DIPI and bis-benzimidazole compounds.

21. The process of Claim 20 in which the dye is in solution.

22. The process of Claim 20 or 21 in which the dye dosage is from about 0.001 μg to about 10 μg per 50 μl culture drop containing said cells or eggs.

23. The process of Claim 18 substantailly as hereinbefore described.

24. Genetic material which has been isolated by the process of any of Claims 18 to 23.

25. A process for transferring genetic mterial into a pronucleus of a living mammalian pronuclear cell comprising:
(a) contacting said cell with a fluorescent dye
(b) incubating until the pronuclei fluoresce, and
(c) transferring the desired genetic material into a pronucleus of a pronuclear cell by micromanipulation techniques.

26. The process of Claim 25 in which said pronuclear cells are bovine or porcine one-cell fertilized eggs.

27. The process of Claim 25 or 26 in which said cells are in culture medium.

28. The process of any of Claims 25 to 27 in which said dye is selected from the group comprising DAPI, DIPI and bis-benzimidazole compounds.

29. The process of Claim 28 in which the dye is in solution.

30. The process of Claim 28 or 29 in which the dye dosage is from about 0.001 μg to about 10 μg per 50 μl culture drop containing said cell.

31. The process of Claim 25 substantially as hereinbefore described.

32. A genetically altered, living mammalian cell having genetic material inserted into it by the process of any of Claims 25 to 31.

33. A living cell or clone derived from the cell of Claim 32.

34. The process of any of Claims 25 to 31 in which said genetic material is isolated by a process comprising:
(a) contacting the cell containing said genetic material with a fluorescent dye,
(b) incubating until said genetic material fluoresces and
(c) removing said genetic material from the cell by micromanipulation techniques.

35. The process of Claim 34 in which said dye is selected from the group comprising DAPI, DIPI and bis-benzimidazole compounds.

36. The process of Claim 34 or 35 in which the dye is in solution.

37. The process of Claim 35 or 36 in which the dye dosage is from about 0.001 μg to about 10 μg per 50 μl culture containing the cell containing said genetic material.

38. The process of Claim 34 substantially as hereinbefore described.

39. Fluorescent mammalian DNA in living mammalian cells selected from the group consisting of germ cells, one-cell fertilized eggs and pre-implantation embryo cells, produced by a process comprising:

(a) contacting the cells with fluorescent dye and

(b) incubating until the DNA fluoresces when exposed to ultraviolet light.

40. The fluorescent mammalian DNA of Laim 39 in which the living mammalian cells used to prepare said fluorescent DNA are bovine or porcine one-cell fertilized eggs and said fluorescent DNA comprises the pronuclei of the cells.

41. The fluorescent mammalian DNA of Claim 39 or 40 in which the fluorescent dye is selected from the group comprising DAPI, DIPI and bis-benzimidazole compounds.

42. The fluorescent mammalian DNA of Claim 41 in which said stain is used in solution.

43. The fluorescent mammalian DNA of Claim 41 or 42 in which the dosage of said stain is from about 0.001 μg to about 10 μg per 50 μl culture containing said cells.

44. A method for generating a photographic representation of spermatozoan movement and tracking pattern comprising:

(a) contacting sperm cells with a fluorescent stain,

(b) incubating until the DNA in the sperm heads fluoresces when exposed to ultraviolet light, and

(c) preparing a time lapse photograph of the fluorescing sperm.

45. The method of Claim 44 in which said flurorescent stain is selected from the group comprising DAPI, DIPI and bis-benzimidazole compounds.

46. The method of Claim 45 in which said stain is in solution.

47. The method of Claim 45 or 46 in which the dosage of said stain is from about 0.001 μg to about 10 μl per 50 μl semen, extended semen or semen-culture medium suspension.

48. A method for the comparative assessment of spermatozoan motility and tracking pattern comprising studying a photographic representation made by the method of Claim 44 in order to determine the qualitative or quantitative aspects of said tracking pattern.

49. The method of Claim 44 substantially as hereinbefore described.

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